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14. ABSTRACT

During the second year of funding, collaborative experiments have demonstrated that monocytes collaborate with MSC in inducing STAT3-dependent drug resistance in neuroblastoma (Task 1), that S1P/S1PR1 contributes to a sustainable STAT3 activation leading toward increased survival (Task 2), and that Jak2 deletion/inhibition prevents drug resistance (Task 3). Experiments aimed at examining the effect of small pathway inhibitors suggest that inhibition of Jak2, MEK and S1PR1 all contribute to prevent drug resistance, but that inhibition of multiple pathways may be required (Task 4). Experiments aimed at examining the role of IL-6 clearly demonstrate that although IL-6 is involved in STAT3-mediated drug resistance, it is not necessary as STAT3 is activated in IL-6 KO mice and tumors develop in IL-6 KO mice crossed with NB-Tag mice (Task 5). As a result, Task 6 which focused on targeting IL-6 has been abandoned. Task 7 has now been initiated using a combination of S1PR1 and Jak2 inhibitors *in vitro* and *in vivo*.

15. SUBJECT TERMS
Drug resistance, tumor microenvironment, neuroblastoma, JAK2, STAT3

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INTRODUCTION:

Neuroblastoma is the most common extra cranial solid tumor of childhood. Approximately 45% of children with neuroblastoma have aggressive tumors, nearly all of which are metastatic when diagnosed. This group includes patients with metastatic disease who are diagnosed at any age with MYCN-A tumors and patients older than 18 months of age with MYCN-non amplified (NA) tumors. During the past 20 years, long-term survival has steadily improved to 40% with increasing intensity of non-specific cytotoxic induction and consolidation therapy, followed by 13-cis-retinoic acid and anti-GD2 antibody immunotherapy of residual disease. It has become increasingly clear that tumor cells that may not be able intrinsically to resist therapeutic insults can acquire these properties as the result of specific interactions with the microenvironment. Although initially transient and reversible, this type of therapeutic resistance promotes the selection of surviving cells that have acquired permanent resistance. In 2005, the DeClerck laboratory identified the production of IL-6 by bone marrow mesenchymal stem cells as a major mechanism promoting osteolytic bone metastasis in neuroblastoma. In 2009 the laboratory demonstrated that in addition to promoting bone metastasis, IL-6 also promotes neuroblastoma cell survival and resistance to cytotoxic drugs. In collaboration, Drs. Seeger, Asgharzadeh and DeClerck demonstrated that not only MSC but also monocytes are a source of IL-6 in the tumor microenvironment of primary neuroblastoma tumors. In collaboration with Dr. Yu, partnering PI on this application, the DeClerck laboratory obtained data demonstrating that STAT3 plays a pivotal role in IL-6-mediated drug resistance in neuroblastoma.

BODY-SCOPE OF WORK:

Task	Sp. Aim	Subtask	Performed by	Site
1. Cooperation between monocytes and tumor cells in IL-6/sIL-6R/STAT3-induced EMDR	1a	Drug sensitivity screen in co-cultures with monocytes	DeClerck/Seeger	CHLA
	1a	Effect of inhibitors of IL-6R/Jak2/STAT3 inhibitors	DeClerck/Seeger	CHLA
	1a	Analysis of survival and apoptotic proteins by Western blot, FACS	DeClerck/Seeger	CHLA
	1a	Co-cultures of fresh neuroblastoma cells-fresh bone marrow monocytes	Seeger	CHLA
2. Role of S1P on STAT3 activation and drug resistance	1b	Effect of IL-6, sIL-6R, and S1P on STAT3 activation, survival and drug resistance	Yu	COH
3. Determine the impact of S1PR1/JAK2/STAT3 signaling in monocytes to drug resistance	1c	Murine NBT2 neuroblastoma cells and human NB cells co-cultured with mouse and human monocytes in which S1PR1 is KO or KD	Yu/DeClerck	COH/CHLA
4. Determine whether S1PR1 and JKA2 are effective targets to block tumor cell-monocyte crosstalk	1d	Co-cultures of NB cells and monocytes in the presence of inhibitors of IL-6, JAK2 and S1PR1 and tested for drug resistance	Yu/DeClerck	CHO/CHLA
5. Effect of IL-6 in tumor and host cells on response to chemotherapy	2a	Breeding to obtain double transgenic homozygous IL-6 null	Asgharzadeh	CHLA
	2a	Imaging and monitoring for tumor development and tumor analysis by histology and TLDA microarrays	Asgharzadeh	CHLA
	2a	Treating NB-Tag mice and NB-Tag/IL-6 KO with cyclophosphamide and topotecan.	Asgharzadeh	CHLA
6. Contribution of bone marrow-derived IL-6 to response to therapy	2b	Transplantation of NB-Tag IL-6 -/- mice with WT bone marrow	Yu/Asgharzadeh	COH/CHLA
	2b	Transplantation into NB-Tag mice, treatment with Poly I:C and monitoring of tumor development	Yu/Asgharzadeh	COH/CHLA
	2b	Effect on drug response: mice will be treated with cyclophosphamide/topotecan and monitored for response	Yu/Asgharzadeh	COH/CHLA

7. Develop strategies that can be translated into clinical trials to overcome EMDR	3a	Testing tocilizumab in SCID mice implanted with human NB cells and monocytes	DeClerck/Seeger	CHLA
	3a	Testing AZD 1480 in SCID mice implanted with human NB cells and monocytes	DeClerck/Seeger/Yu	CHLA/COH

KEY RESEARCH ACCOMPLISHMENTS:

Task 1. Cooperation between monocytes and tumor cells in IL-6/sIL-6R/STAT3-induced EMDR: This task was completed last year and the manuscript published in *Cancer Research* in 2013 (Ara *et al*. 2013).

Task 2. Role of S1P on STAT3 activation and drug resistance: This task has been performed by our collaborator at City of Hope, Dr. Hua Yu. She has shown the importance of S1PR1-STAT3 in EMDR in neuroblastoma. In a published manuscript (Yang *et al*., *Cancer Biology & Therapy* 2012), she demonstrated that sorafenib inhibits endogenous and IL-6/S1P-induced JAK2-STAT3 signaling in human neuroblastoma, associated with growth suppression and apoptosis. In order to understand the mechanism of drug resistance and to develop targeted strategies for combating drug resistance, she has generated etoposide-resistant human neuroblastoma cells and demonstrated that etoposide-resistant human cells have highly elevated S1PR1 expression and STAT3 activity. A manuscript (Priceman, Lifshitz *et al*., *S1PR1-STAT3 signaling is critical for neuroblastoma chemo-resistance*) is in preparation.

Task 3. Determine the impact of S1PR1/JAK2/STAT3 signaling in monocytes to drug resistance: We have generated three strains of mice homozygous floxed for the gene of interest (S1PR1, JAK2 or STAT3), and hemizygous for Rosa26-CreERT2 (Cre recombinase - estrogen receptor T2). We have begun to test the impact of S1PR1/JAK2/STAT3 signaling in myeloid lineage to drug resistance using co-culture of murine NBT2 neuroblastoma cells and murine S1PR1 and JAK2 knockout bone marrow-derived monocytes. Preliminary data indicates that co-culture of NBT2 cells with JAK2 deficient BM-derived myeloid cells potentiates the cytotoxicity of etoposide and decreases levels of pSTAT3 and S1PR1 expression in tumor cells (Fig.1).

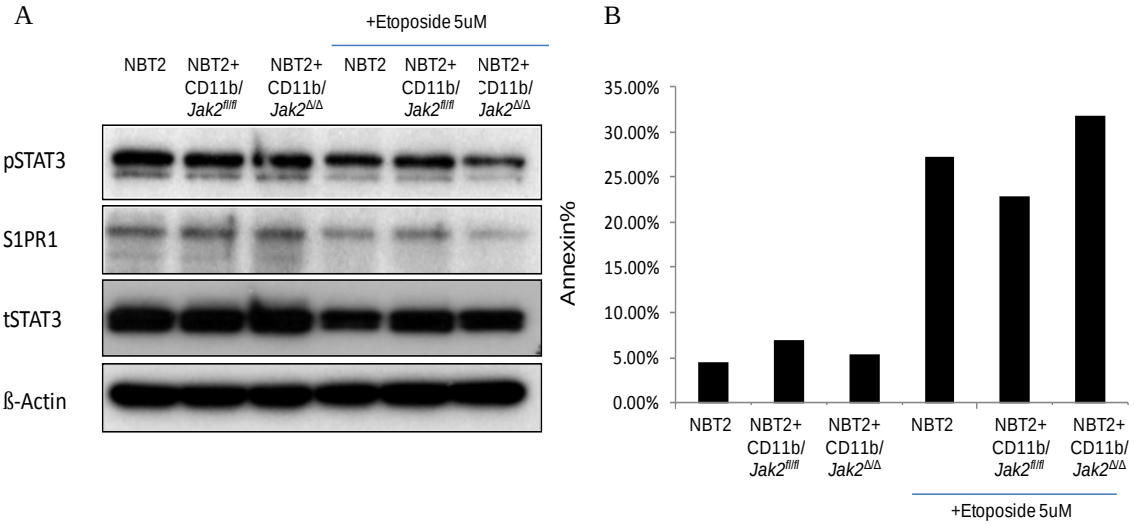

Figure 1. Deletion of JAK2 in BM-derived myeloid cells decreases STAT3-mediated survival of neuroblastoma cells. A. *Representative western blot of pSTAT3 and S1PR1 as detected in NBT2 cells co-cultured with BM-derived myeloid cells derived from Jak2$^{fl/fl}$ or JAK2$^{fl/fl}$CreERT2 mice.* ***B.*** *The co-cultures were treated with etoposide (5 μM) for 24 h. Apoptosis was determined by Annexin V staining and FACS analysis.*

Task 4. Determine whether S1PR1 and JAK2 are effective targets to block tumor cell-monocyte crosstalk. We have begun to test the effect of STAT3 blocking on drug resistance induced in co-cultures of human tumor cells and mesenchymal stromal cells. We have used a combination of inhibitors of Jak2 and ERK1/2 as we found activation of both pathways when neuroblastoma cells were exposed to the conditioned medium of co-cultures of human MSC and NB cells. We tested ruxolitinib (a Jak2 inhibitor) and trametinib (an MEK inhibitor). In these experiments NB cells were cultured either in the presence of their own medium or in the presence of conditioned medium obtained from 48 hrs of co-cultures of NB and MSC (or skin fibroblasts as control). The data demonstrated that MSC increase the resistance of NB cells to chemotherapeutic agents like etoposide or melphalan (Figure 2A) and stimulate in NB cells the activation of not only STAT3 as previously

reported {Ara, 2013 #6449} but also ERK1/2 (Figure 2B). We then tested the effect of ruxolitinib (a Jak2 inhibitor as Jak2 activation is upstream of STAT3 activation), and trametinib (GSK 2118436), a selective MEK inhibitor, for their effect on drug sensitivity. This experiment (Fig. 2C) indicated that the addition of ruxolitinib or trametinib alone had no inhibitory effect on the activity of the CM of educated MSC in increasing resistance to etoposide as the right shift of the dose-response curve for etoposide was maintained whereas the curve shifted back to the left when trametinib and ruxolitinib were both added at 1 µM, a concentration where they had little or no toxicity on their own (not shown). The data thus indicate that both STAT3 and ERK1/2 contribute to EMDR induced by MSC drug resistance.

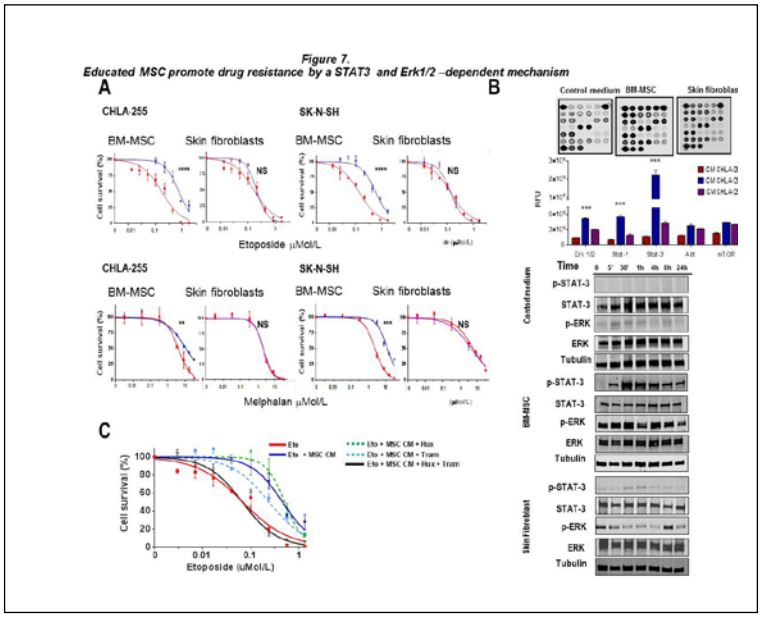

Figure 2. A combination of Jak2/STAT3 and MEK/ERK1/2 inhibition restores the sensitivity of NB cells to etoposide in the presence of MSC CM. A. *CHLA-255 and SK-N-SH NB-cells cultured in the presence of CM from their own medium (reconstituted 50/50 with fresh medium; Red line) or medium from educated MSC or skin fibroblasts (Blue line), were treated with etoposide or melphalan at the indicated concentrations. After 48 hr. cells were examined for viability by CellTiter-Glo bioluminescence. The data represent the mean (±SD) percentage of luminescence from control in two experiments conducted in triplicate.* ***B.*** *CHLA-255 cells were cultured in conditions described in* ***A*** *but after 1 hr. cells were lysed and examined for the presence of phosphorylated proteins by phosphor dot-blot (Top) or Western blot analysis (Bottom).* ***C.*** *CHLA-255 were cultured in the presence of CM from educated MSC (blue line) or their own medium (red line) and treated with indicated concentrations of ruxolitinib (dotted green) or trametinib (dotted blue) or the combination (solid black) before being exposed to etoposide. Cells were examined after 48 hr. for viability by CellTiter-Glo (Promega). The data represent the mean percent luciferase activity from control of triplicate samples.*

In vitro data showed anti-tumor therapeutic effect in NBT2 cells treated with S1PR1 and JAK2 inhibitors (FTY720 and AZD1480, respectively) (Fig. 3). Currently, we are testing the effectiveness of S1PR1 and Jak2 inhibitors in combination with chemotherapeutic agents *in vitro* and *in vivo* using NSG mice injected with CHLA-255Fluc cells and NB-Tag mice.

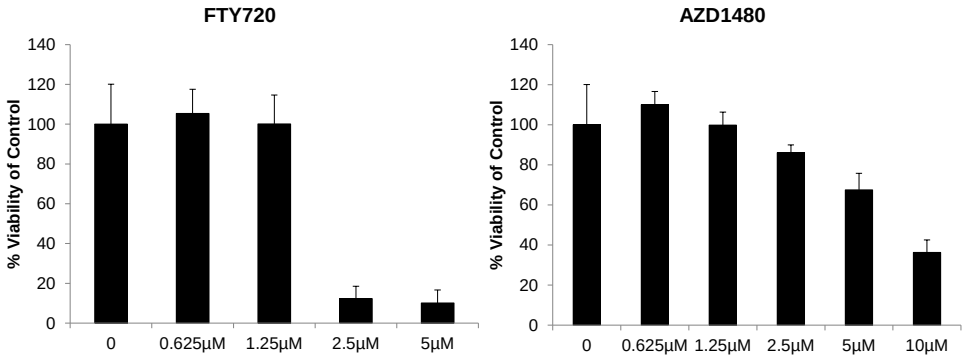

Figure 3. Effects of specific inhibition of S1PR1 and Jak2 in NBT2 cell line. *Cell viability was assayed with an MTS assay (Promega) 24 h post treatment with the indicated concentrations of FTY 720 and AZD1480.*

Task 5. Effect of IL-6 in tumor and host cells on response to chemotherapy: Our collaborator Dr. Asgharzadeh has generated NB-Tag mice in an IL-6 KO background. He found that NB tumors in these mice develop at the anticipated rate seen in WT NB-Tag mice, indicating that lack of IL-6 does not affect tumor initiation and growth (Fig. 3A). While IL-6 production in these tumors has been demonstrated to occur during tumor growth and produced by macrophages, the growth promoting effects of these macrophages are evident even in the absence of IL-6 (Fig. 3B). Interestingly, an analysis of these tumors for pSTAT3 by Western blot and immunohisto-chemistry revealed the presence of pSTAT3, suggesting the presence of alternate pathways of activation. The loss of IL-6 also does not alter the response of tumors to treatment with chemotherapy regimen (5 days of cyclophosphamide and topotecan), and time to regrowth is similar in both WT and IL-6 KO animals (Fig. 3D).

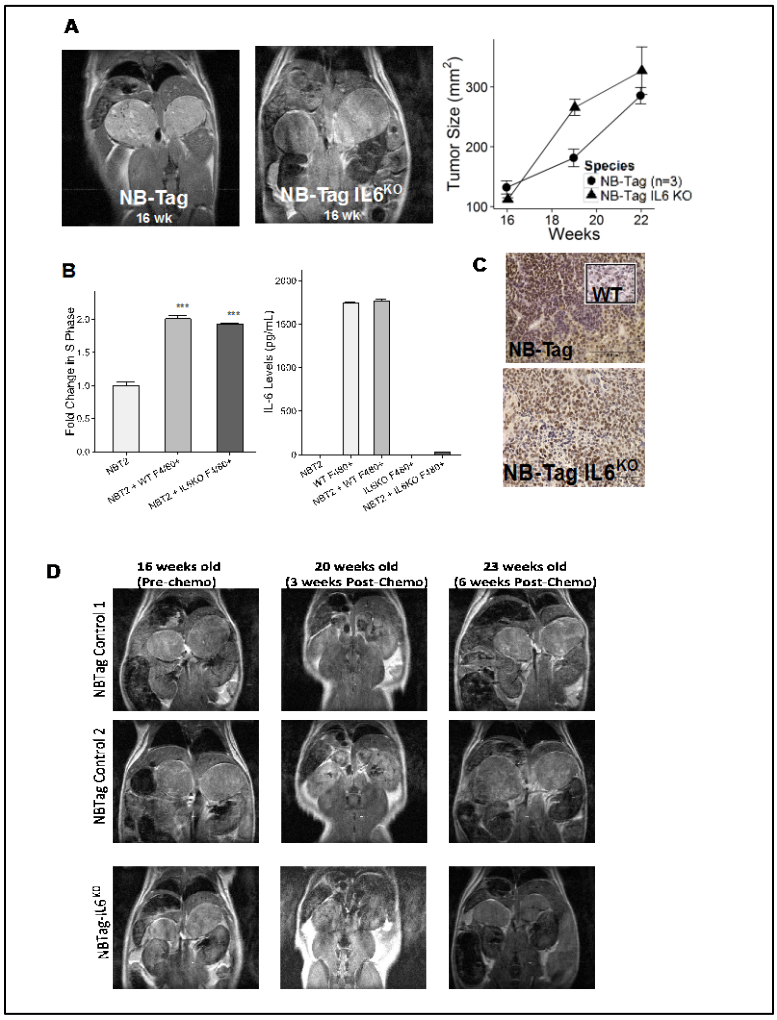

Figure 4: *Effect of IL-6 knockout crossing with NB-Tag mice.* ***A.*** *MRI of abdominal cavity of representative mice from NB-Tag and NB-TagxIL-6^{KO}at 16 weeks of age, and growth rate of the two models over time (n=3-4 mice per group)* ***B.*** *Proliferation index as measure by BrdU incorporation shows no difference between tumors co-cultured with WT or IL-6^{KO} macrophages. The co-culture experiments also demonstrated a lack of IL-6 production in co-cultures between NB-Tag cell line and IL-6^{KO} macrophages.* ***C.*** *Immunohistochemical analysis of adrenal gland reveals no pSTAT3 expression while both NB-Tag and NB-TagxIL-6^{KO} express pSTAT3 suggesting alternative pathways of activation independent of IL-6 production.* ***D.*** *Representative MRI images of NB-Tag and NB-Tag/IL-6^{KO} pre-chemotherapy, post 3 and 6 weeks of chemotherapy.*

Task 6. Contribution of bone marrow-derived IL-6 to response to therapy: Based on findings from Task 5, it would be illogical to perform wild-type bone marrow transplantation into NB-Tag/IL-6 KO mice, as we have demonstrated a growth pattern similar to WT NB-Tag mice. However, our data also provided insight into the possibility that multiple pathways may converge to phosphorylate STAT3, and removal of IL-6 alone may be insufficient to overcome EMDR. Future experiments for this task will assess the significance of STAT3 in this model following similar strategies outlined in Task 5 and 6.

Task 7. Develop strategies that can be translated into clinical trials to overcome EMDR: Last year we tested the effect of tocilizumab in mice implanted with human NB tumor cells and human monocytes. These experiments were not conclusive and although they initially showed some effects, repeated experiments failed to show a statistically significant effect. This approach was abandoned. However, because *in vitro* data showed anti-tumor therapeutic effect in CHLA-255 cells induced by S1PR1 inhibitor (FTY720) alone or in combination with etoposide (Fig. A), we tested this approach *in vivo* and preliminary data showed significant decrease in tumor weight in NSG mice injected with CHLA-255Fluc cells following FTY720 treatment (Fig. 5B). Ongoing studies try to determine the therapeutic impact of S1PR1 and JAK2 inhibitors in combination with chemotherapeutic agents in NSG mice implanted with CHLA-255Fluc cells and in mouse model of neuroblastoma (NB-Tag mice).

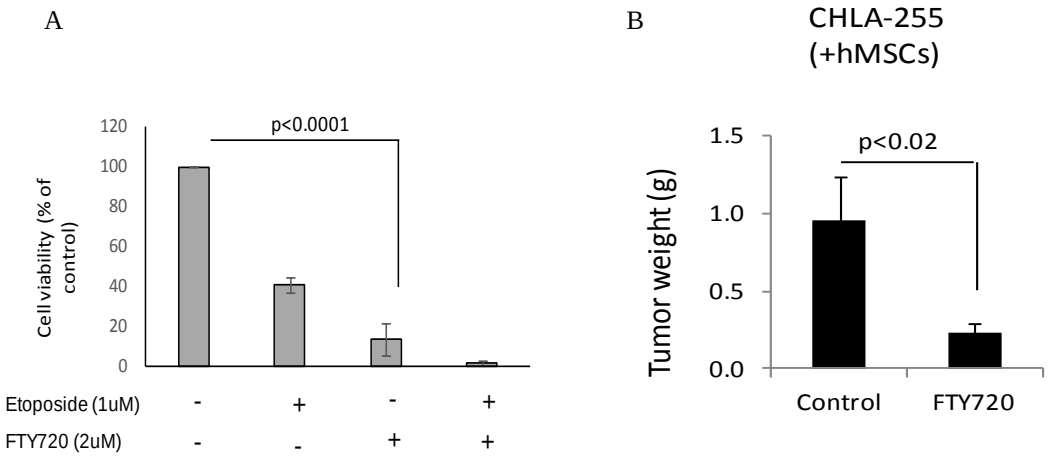

Figure 5. Effect of S1PR1 antagonist FTY720 on human neuroblastoma cells. A. *CHLA-255 neuroblastoma cell line was pretreated with FTY720 (2 μM) following etoposide (1 μM) treatment for 24 h. Cell viability was determined using MTS assays. Shown are the mean of three independent experiments, each performed in triplicates.* ***B.*** *NSG mice were injected subcutaneously with CHLA-255 cells and hBM-MSC (100:1). Week after implantation, mice were treated with 5 mg/kg FTY720 or vehicle control by intraperitoneal injection daily. After 12 days tumors were excised, measured and weighed. Data are mean ± SEM.*

We have also initiated a series of experiments testing the therapeutic impact of targeting S1PR1 to overcome EMDR in mouse models of neuroblastoma (NB-Tag mice). Donor BMC from CreERT2/S1PR1$^{-/-}$ and appropriate littermate controls were harvested from femurs and tibias, and injected intravenously in lethally irradiated NB-Tag recipient mice. The preliminary data showed no significant difference in tumor size (after 5 weeks) in both of the groups. We are planning to repeat this experiment in combination with chemotherapeutic agents. In another set of experiments we will overexpress S1PR1 in NBT2 cells and inject them into CreERT2/S1PR1$^{-/-}$ mice (our preliminary data showed that NBT2 cells either failed to engraft or regressed in CreERT2/S1PR1$^{-/-}$ mice).

REPORTABLE OUTCOME:

Ara, T., Nakata, R., Shimada, H., Buettner, R., Groshen, S.G., Ji, L., Sheard, M., Yu, H., Jove, R., Seeger, R.C. and DeClerck, Y.A. Critical role of STAT3 in interleukin-6-mediated drug resistance in human neuroblastoma. Cancer Res, 73:3852-3864, 2013.

CONCLUSIONS:

Over the last year we have generated data that brought additional light to the mechanism by which the TME protects tumor cells from drug-induced apoptosis. In particular we show:

1. That MSC and monocytes collaborate in part by activating STAT3 and ERK1/2 in tumor cells and promote survival and drug resistance.
2. STAT3 activation – although downstream of IL-6 – is activated by other pathways including S1PR1 mediated signaling.
3. Inhibitors of S1PR1 such as FTY720 prevent EMDR *in vitro* and have anti-tumor activity *in vivo.*